QuantiGene® ViewRNA Plate-Based Assay

(for adherent cells)

User Manual

P/N 16820 Rev B 091009

Panomics

Panomics, Inc.

QuantiGene ViewRNA Plate-Based User Manual

Copyright

© Copyright 2009, Panomics, Inc. All rights reserved.

Trademarks

QuantiGene is a registered trademark exclusively licensed to Panomics, Inc. All other trademarks belong to their respective owners.

Citing QuantiGene ViewRNA in Publications

When describing a procedure for publication using this product, please refer to it as the QuantiGene ViewRNA assay.

If a paper cites a QuantiGene ViewRNA product and is published in a research journal, the lead author(s) may receive a travel stipend for use at a technology conference or tradeshow by sending a copy of the paper to our technical support group at techsupport@panomics.com or via fax at (510) 818-2610.

Disclaimer

Panomics, Inc. reserves the right to change its products and services at any time to incorporate technological developments. This manual is subject to change without notice.

Although this manual has been prepared with every precaution to ensure accuracy, Panomics, Inc. assumes no liability for any errors or omissions, nor for any damages resulting from the application or use of this information.

Contacting Panomics

U.S Corporate Headquarters

Panomics, Inc.
6519 Dumbarton Circle
Fremont, CA 94555
Toll Free: 877 PANOMICS (1.877.726.6642)
Direct: 1.510.818.2600
Fax: 1.510.818.2610
Email: info@panomics.com
Email: orders@panomics.com
Email: techsupport@panomics.com

European Headquarters

Panomics Srl
Via Sardegna 1
20060 Vignate-Milano (Italy)
Tel: +39.02.95.360.250
Fax: +39.02.360.992
Email: info_europe@panomics.com
Email: order_europe@panomics.com
Email: techsupport_europe@panomics.com

Asia Pacific Headquarters

Panomics, Inc.
16F Gemdale Plaza Tower A, No. 91
Jiango Road, Beijing 100022
P.R. China
Tel: +86.10.59208157
Fax: +86.10.59208111
Email: info_asia@panomics.com
Email: order_asia@panomics.com
Email: techsupport_asia@panomics.com

Contents

Section I: Introduction 1
About This Manual 1
QuantiGene ViewRNA Assay Basics 1
How It Works 1
Section II: Required Materials 2
QuantiGene ViewRNA Reagent System 2
QuantiGene ViewRNA Plate-based Assay Kit Components 2
QuantiGene ViewRNA Plate-Based Signal Amplification Kits 2
QuantiGene ViewRNA Probe Sets 3
Required Materials Not Provided 3
Section III: Recommendations for Experimental Design and Assay Optimization 6
Overview 6
Selecting the Appropriate Plate type 6
Replicates 6
Assessing Background for New Projects 6
Using New Probe Sets 7
Optimizing Assay Conditions for a New Cell Type 7
Optimizing Duplex Assay Formats 8
Section IV: Assay Procedures for Automated or Batch Processing (Residual Volume Method) 9
Overview 9
Using a Residual Volume 9
Important Procedural Notes 10
Preparing Reagents 10
Fixing Cells and Treating with Protease 12
Performing Hybridizations 13
Section V: Troubleshooting 18
Troubleshooting No or Weak Signals 18
Variable Staining Within a Sample 19
High Background on Entire Plates 19
High Background Within Cells 20
High Number of Dots Appear where Cells are Absent 20
No DAPI or Dots 20
Appendix I: Liquid Handling Recommendations 21
Fluidic Handling Systems 21
Minimum Requirements 21
Setup Recommendations 21
Wash Protocol Settings for BioTek ELx405 Select 21

Appendix II: Procedure for Dehydrating Cells for Storage at –20 °C, or for Shipping . 23
About Dehydration Procedure 23
Procedure 23
Appendix III: 96- and 384-Well Imaging-Compatible Plates 24
Compatible Plates 24
Appendix IV: Optimizing Assay Conditions for a New Cell Type 25
Initial Optimization and Assessment of Assay Backgrounds 25
Examples of Optimized Conditions 26
Additional Optimization 27
Appendix V: Alternative Assay Procedure (Manual Processing) 28
Overview 28
Important Procedural Notes 28
Preparing Reagents 28
Fixing Cells and Treating with Protease 31
Performing Hybridizations 31

Section I: Introduction

About This Manual

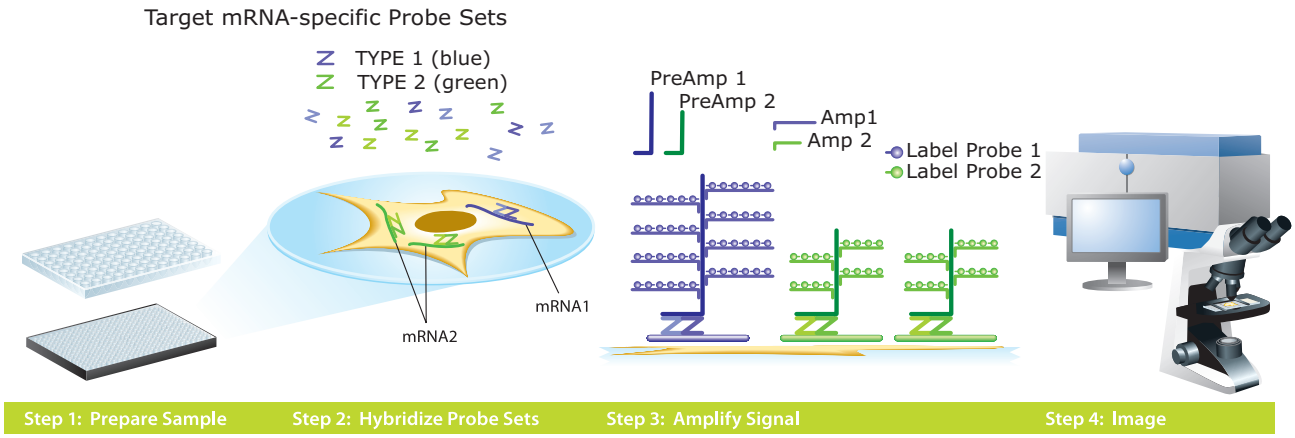
The QuantiGene ViewRNA Reagent System is an *in situ* hybridization method for visualization of one target RNA (at single transcript detection level) and one housekeeping or higher expressing (>1,000 transcripts) target RNA simultaneously within individual cells. This manual provides complete instructions for performing QuantiGene ViewRNA assays for adherent cells in either:

- An automated format for 96- or 384-well plates or
- A manual format for 96-well plates

QuantiGene ViewRNA Assay Basics

Panomics' QuantiGene ViewRNA assay is a novel RNA *in situ* hybridization solution, based on patent-pending Probe Set design and proprietary signal amplification technology. QuantiGene ViewRNA offers single copy RNA sensitivity in individual cells in a multiplex assay format. Signal amplification is predicated on specific hybridization of adjacent Probe Set oligonucleotides to a target RNA (see How It Works below), resulting in excellent signal-to-noise ratios. Independent but compatible signal amplification systems enable simultaneous detection of two RNAs in a single assay.

How It Works



Step 1: Prepare Sample. Adherent cells on a solid surface are fixed and permeabilized.

Step 2: Hybridize Probe Sets. Gene-specific Probe Sets hybridize to target mRNAs. For clarity, only single oligonucleotide pairs are shown, however, a typical Probe Set contains 20 or more oligonucleotide pairs. Each Probe Set TYPE, for example TYPE 1, interacts specifically with a corresponding signal amplification system, for example PreAmp 1, Amp 1, Label Probe 1, to generate signal for visualization.

Step 3: Amplify Signal. Independent but compatible signal amplification systems enable simultaneous detection of multiple RNAs in a single assay. Distinct sets of PreAmp, Amp, and Label Probe molecules are used to detect different target RNAs.

Step 4: Image. Target mRNAs are visualized using standard fluorescence microscopy.

Section II: Required Materials

QuantiGene ViewRNA Reagent System

The QuantiGene ViewRNA Plate-Based Reagent System is comprised of 3 modules (each sold separately):

- QuantiGene ViewRNA Plate-Based Assay Kit
- QuantiGene ViewRNA Plate-Based Signal Amplification Kit
- QuantiGene ViewRNA Probe Sets

QuantiGene ViewRNA Plate-based Assay Kit Components

The components of the QuantiGene ViewRNA Plate-Based Assay Kit and their recommended storage conditions are listed below. This Assay Kit contains the universal reagents for running a QuantiGene ViewRNA assay. This Assay Kit must be used in conjunction with a QuantiGene ViewRNA Plate-based Signal Amplification Kit and appropriate QuantiGene ViewRNA probe sets.

The QuantiGene ViewRNA Plate-based Assay Kit is available in multiple sizes. Refer to the product insert for quantities of individual components supplied. Kits have a shelf life of 6 months from date of receipt.

Component	Description	Storage
Protease	Enzyme in aqueous buffered solution	2-8 °C
Hybridization Buffer A (Hyb A)	Aqueous solution containing formamide and detergent	2-8 °C
Hybridization Buffer B (Hyb B)	Aqueous solution containing formamide and detergent	2-8 °C
Hybridization Buffer C (Hyb C)	Aqueous solution containing detergent	2-8 °C
Protease Stop Buffer	Aqueous buffered solution	15-30 °C
Storage Buffer	Aqueous buffered solution	15-30 °C
Detergent Solution	Aqueous buffered solution containing detergent	15-30 °C
Wash Buffer Component 1 (Wash Comp 1)	Aqueous solution containing detergent	15-30 °C
Wash Buffer Component 2 (Wash Comp 2)	Aqueous buffered solution	15-30 °C
Pre-Hybridization Buffer (PreHyb Buffer)	Aqueous buffered solution	15-30 °C

QuantiGene ViewRNA Plate-Based Signal Amplification Kits

The components of the QuantiGene ViewRNA Plate-Based Signal Amplification Kit and their recommended storage conditions are listed below. There are multiple options for signal amplification in the QuantiGene ViewRNA assay. Depending on the need for single or multiplex assays, the items supplied will differ. PreAmp 1/Amp 1/LP 1 are compatible with TYPE 1 Probe Sets and PreAmp 2/Amp 2/LP 2 are compatible with TYPE 2 Probe Sets. Refer to the product insert for quantities of individual components supplied. Kits have a shelf life of 6 months from date of receipt.

Component	Description	Storage
PreAmplifier (PreAmp 1 and/or PreAmp 2)	DNA in aqueous buffered solution	–20 °C

Amplifier (Amp 1 and/or Amp 2)	DNA in aqueous buffered solution	–20 °C
Label Probe (LP1 and/or LP2)	Fluorescent dye-conjugated oligonucleotides in aqueous buffered solution	–20 °C

QuantiGene ViewRNA Probe Sets

In addition to the Assay Kit and Signal Amplification Kit, QuantiGene ViewRNA Probe Sets specific to your target and housekeeping or higher expressing RNAs of interest must be purchased separately. Probe Sets are available in multiple sizes. Refer to the product insert for details on Probe Set design and specificity. QuantiGene ViewRNA Probe Sets should be stored at –20 °C and have a shelf life of 12 months from date of receipt.

Component	Description	Storage
QuantiGene ViewRNA TYPE 1 Probe Set	RNA-specific oligonucleotides for use with the PreAMP1/Amp1/LP1 Signal Amplification Kit, for visualization of single copy RNAs (1 copy/cell)	–20 °C
QuantiGene ViewRNA TYPE 2 Probe Set	RNA-specific oligonucleotides for use with the PreAMP2/Amp2/LP2 Signal Amplification Kit, for visualization of high abundance RNAs such as 18S, ACTB or GAPD (more than 1000 copies/cell)	–20 °C

Visit www.panomics.com for the most up-to-date listing of available QuantiGene ViewRNA Probe Sets. By Request QuantiGene ViewRNA Probe Sets can be designed and synthesized at no additional cost. Please provide the accession number (including version or gi number) or RNA sequence with your By Request order.

Required Materials Not Provided

Other materials required to perform the QuantiGene ViewRNA assay that are not included in the Plate-Based Assay Kit are listed here.

Table 1 Required materials not provided:

Required Material	Source	Part Number or Model
Nuclease-free water	Major laboratory supplier	
100% Ethanol (optional)	Sigma Aldrich or equivalent	459844
10X PBS	Ambion or equivalent	AM9625
37% Formaldehyde **! WARNING ! Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.**	Fisher Scientific	F79-1
96- or 384-well imaging-compatible tissue culture plates	See “Appendix III: 96- and 384-Well Imaging-Compatible Plates” on page 24.	
QuantiGene Incubator Temperature Validation Kit	Panomics	QS0517

Table 1 Required materials not provided:

Required Material	Source	Part Number or Model
Incubation oven with the following specifications: ♦ 40 ± 0.5 °C that does not deviate by more than 1 °C when opening/closing ♦ Uniform temperature throughout the entire incubator ♦ Prefer 90% humidity ♦ Automation-friendly	Panomics or Liconic or Thermo Cytomat models recommended for automation	QS0700 (120V) QS0710 (220V) QS0701 (120V) QS0711 (220V)
DAPI DNA stain solution **! WARNING ! DAPI is a possible mutagen. Avoid contact with skin and mucous membranes.**	Invitrogen or equivalent	D21490
Epi-fluorescence microscope that meets the following specifications: ♦ 20-40X objective ♦ 100 Watt mercury bulb source ♦ Filter sets for: – Ex 358 nm/Em 461 nm (DAPI) – Ex 495 nm/Em 519 nm (FITC) – Ex 556 nm/Em 573 nm (Rhodamine)	Olympus or equivalent	IX71
Imaging system with the following features: ♦ 20x or 40x objective ♦ Uses standard 96- or 384-well plates ♦ Accepts Filter sets for DAPI, Fluorescein, and Rhodamine wavelengths ♦ Autofocus ♦ Broad excitation wavelength (350-600 nm) ♦ Cooled CCD	Applied Precision, Inc. Thermo Scientific GE Healthcare MDS Analytical or equivalent	CellWoRx Cellomics Array Scan InCell 1000 ImageXpress Micro
A fluidic dispenser and plate washer, sold separately or together as outlined below		
Fluidic handling system 96- or 384-channel pipet that meets or exceeds the following specifications: ♦ 15-70 µL ± 5% volume dispensing ♦ 5-20 µL/sec rate of aspiration/dispense ♦ Replace or wash tip between dispensing	Thermo Scientific Matrix Beckman Coulter Agilent Technologies (Velocity11) or equivalent	PlateMate 2 x 3 Biomek FX or NX Bravo

Table 1 Required materials not provided:

Required Material	Source	Part Number or Model
Plate washer that meets or exceeds the following specifications: ♦ 30-200 µL ± 5% volume ♦ 96 or 384 channels ♦ Angle-dispensing tip ♦ Plate stacker ♦ Automation capable ♦ Minimal dead volume	BioTek	ELx405 model with high throughput pump option
Combination washer dispenser	BioTek	ELx406

Section III: Recommendations for Experimental Design and Assay Optimization

Overview

In this section, we provide recommendations for experimental design and optimizing assay conditions.

Selecting the Appropriate Plate type

Firm adherence of cells to the plate is essential for a successful assay. Selection of the assay plate should be based on the following criteria:

Strong Cell Attachment

Better adherence to the plate means that fewer cells will be lost during the processing, resulting in better assay precision. The goal should be 80-95% confluence from start to finish of the assay procedures.

Plastic-bottomed cell culture plates are typically better for cell attachment than glass-bottomed plates. However, plastic-bottomed plates tend to exhibit higher (2-4X) backgrounds.

Glass-bottomed plates typically have superior optical qualities and lower background. They can be coated with extracellular matrices such as poly-D-lysine, MatriGel or collagen to enhance cell attachment.

Imaging System Compatibility

Imaging platforms often have limitations on plate types compatible with their systems. Please ensure the plates used are compatible with your imaging system.

Low Background with the QuantiGene ViewRNA Assay

Plates must have acceptable background levels to achieve required sensitivity and assay precision. Some plate coatings may induce non-specific binding of the QuantiGene ViewRNA amplification system resulting in high backgrounds. To assess plate background, run a no-cells control described below in “Assessing Background for New Projects” on page 6.

Automation-friendly

Use a plate type that follows the ANSI-recommended specifications. The plate should also have a lid to control evaporation.

Replicates

Perform all assays in duplicate or triplicate.

Assessing Background for New Projects

It is important to assess the source of background signals when running a new assay. The results can be evaluated using either a microscope or imaging system. We recommend running the following controls:

- *No Cells*. Designate at least 2-3 wells that contain no cells and undergo the entire assay procedure. This control enables you to assess the background associated with non-specific binding of signal amplification reagents to the well and/or well coating. There should be a low, uniform intensity across the well. The intensity for these wells should be lower than wells that contain cells only and wells that contain no probe sets. The appearance of patchy areas with enhanced intensity or many strong

individual dots indicates a problem with the assay. See “Section V: Troubleshooting” on page 18 for more information.

- *Cells Only*. Designate at least 2-3 wells that undergo only the cell staining with DAPI. This control enables you to assess the amount of autofluorescence the cells are contributing to assay background.
- *No Probe Set(s)*. Designate 2-3 wells that undergo the entire assay procedure with the probe sets omitted. This control enables you to assess the non-specific binding of amplification reagents to the cell. There should be less than 1 strong dot/10 cells.
- *Negative Control Probe Set*. Designate 2-3 wells that contain Probe Set targeting a gene that is not expressed in the target cell. This control enables you to assess the specificity of the assay. For example, using a TYPE 1 Probe Set designed to detect the sense strand of 18S RNA. There should be less than 1 strong dot/10 cells.
- *Positive Control Probe Set*. Designate 2-3 wells that contain a Probe Set targeting a housekeeping gene (such as GAPD), or another gene that is expected to be expressed. There should be distinct dots in the cytosol of the cells. The background should be comparable to the no-Probe Set control.

Note A strong dot is defined as exhibiting a measurement of 2.5X signal-to-background ratio. Signal to background is calculated by taking the maximum signal of a dot and dividing by the average background signal outside of the cell.

Using New Probe Sets

When using a Probe Set for the first time, we recommend running the Probe Set in both a singleplex and multiplex assay format.

Optimizing Assay Conditions for a New Cell Type

When working with a new sample type, optimization of cell density, formaldehyde fixation and Protease digestion are key factors. In the initial experiment, we recommend that you start with a singleplex assay, 80-95% cell density, a 30 minute fixation time and a 10 minute incubation time with Protease dilutions 1:1,000, 1:2,000, 1:4,000, and 1:8,000. This approach should work for most sample types. In some cases, it may be necessary to evaluate additional cell densities and fixation times of 15 and 60 minutes. Under fixation and over treatment with Protease will result in high cell loss. Cell loss can be checked using a bright field microscope. See “Appendix IV: Optimizing Assay Conditions for a New Cell Type” on page 25 for sample plate layouts for the optimization conditions.

The selection criteria for the optimal fixation and Protease treatment conditions are the balance of cell loss and maximum signal to background. When visualizing the plate under a fluorescent microscope or in an imaging platform, the following criteria should be used to select the optimal assay conditions:

- Least cell lost. The majority of cell loss typically occurs after the Protease treatment. We recommend that you verify the cell density after the Protease treatment using a bright field microscope. If the cell confluence is greater than 50%, proceed with the assay.
- Lowest background inside and outside cells in the negative control wells (low number of dots, less than 1 strong dot/10 cells, and low, even background).
- Highest signal/background ratio of dots inside the cell.
- Best assay precision between replicate wells and plates for cell number, low background and signal/background ratio.

Optimizing Duplex Assay Formats

To detect two RNAs in a multiplex assay, you must use one, TYPE 1 Probe Set and one, TYPE 2 Probe Set.

IMPORTANT Never combine two, TYPE 1 Probe Sets or two, TYPE 2 Probe Sets in a multiplex assay.

Note TYPE 1 Probe Sets hybridize to the PreAMP/AMP/LP 1 signal amplification system, and can be used to visualize single copy RNAs.

Note TYPE 2 Probe Sets hybridize to PreAmp/Amp/LP 2, and can be used to visualize high abundance (> 1,000 copies/cell) RNAs such as 18S, ACTB, or GAPD.

Section IV: Assay Procedures for Automated or Batch Processing (Residual Volume Method)

Overview

The following procedures can be completed in one long day, or split between 2 days.

IMPORTANT These procedures are optimized for using 96- or 384-well multichannel pipettor and microplate washer. It is not ideal for manual microplate manipulation. See "Appendix V: Alternative Assay Procedure (Manual Processing)" on page 28.

IMPORTANT Before starting this procedure, carefully review the recommendations in "Section III: Recommendations for Experimental Design and Assay Optimization" on page 6.

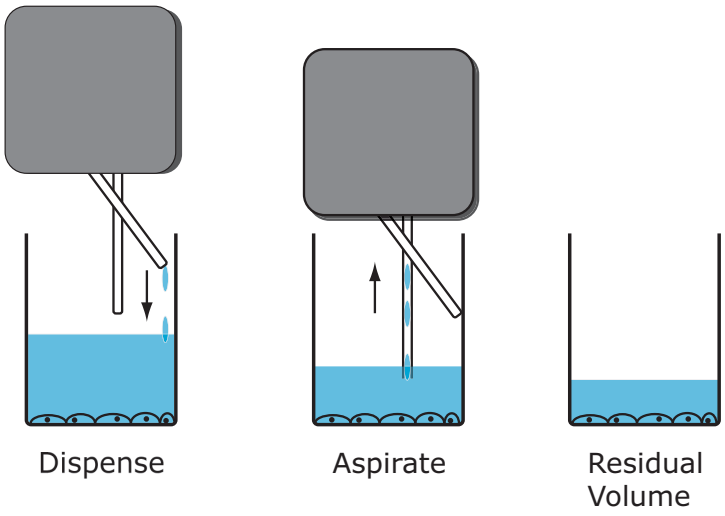
Assay Workflow

- Sample preparation. Adherent cells must be plated in 96- or 384-well format and allowed to firmly adhere to the plate before starting the Assay Procedure.
- Preparing reagents
  - Prewarm reagents
  - Prepare working reagents
- Fix cells (optional stop point)
- Permeabilize cells and digest with Protease
- Perform hybridizations
  - Hybridize Probe Sets (optional stop point)
  - Sequentially hybridize PreAmp(s), Amp(s), and LP(s)
  - Counter stain with DAPI (optional stop point)
  - Analyze samples on imaging system

Using a Residual Volume

To minimize cell detachment from the bottom of the plate, the following procedures leave a residual volume in each well, at each step. At the end of each step there is always a minimum volume of 15 µL/well in a 384-well format and 30 µL/well in a 96-well format. See "Appendix I: Liquid Handling Recommendations" on page 21 for more information.

This figure illustrates what residual volume is, the volume remaining after aspiration.



All quantities of buffers used in these procedures take into account the presence of this residual volume. If you must increase the residual volume, to further minimize cell disruption, adjust the quantities of the assay reagents proportionally so that they match the residual volume.

Note If making this procedural modification, additional assay reagents may need to be purchased.

IMPORTANT If you are working in 96-well formats and are using manual pipetting, please refer to "Appendix V: Alternative Assay Procedure (Manual Processing)" on page 28 for a procedure that does not utilize residual volumes.

Important Procedural Notes

- Hybridization reactions must be carried out at 40 ±1 °C. Verify and monitor oven temperature using the QuantiGene Incubator Temperature Validation Kit.
- Protect samples from light during the Label Probe hybridization and all subsequent steps.
- Before opening reagents supplied in microfuge tubes, briefly centrifuge to collect contents at the bottom of the tube.
- All Hyb Buffers stored at 4 °C must be prewarmed to 40 °C for 30 minutes to redissolve any precipitates.
- If necessary, Probe Set/PreAmp/Amp/LP reactions can be left in wells at room temperature for 30 minutes before placing at 40 °C.
- If necessary, Probe Set/PreAmp/Amp/LP reactions can be left in wells at room temperature for 30 minutes before washing steps.
- PreHyb Buffer must be kept at 40 °C at all times during the procedure to prevent precipitation.

Preparing Reagents

To prepare reagents:

Step	Action
1	Thaw/warm the following reagents: ♦ Prewarm PreHyb, Hyb A, Hyb B, and Hyb C Buffers to 40 °C for 30 minutes. ♦ Thaw Probe Sets, PreAmp (or PreAmps), Amp (or Amps), and LP (or LPs) and place on ice. **IMPORTANT** PreHyb Buffer must remain at 40 °C during the entire procedure to prevent precipitation.
2	Prepare the appropriate volume of the following reagents: ♦ 1X PBS ♦ 8% Formaldehyde Solution in 1X PBS (prepare fresh each time) **! WARNING ! Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.**

To prepare reagents: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>3</td><td>Prepare Wash Buffer. For example, to a 1,000-mL graduated cylinder, add the following in this order:
♦ 800 mL nuclease-free water
♦ 3 mL Wash Buffer Comp 1
♦ 5 mL Wash Buffer Comp 2
♦ Adjust volume to 1 liter with nuclease-free water
Scale preparation according to the number of plates to be processed.</td></tr>
<tr><td>4</td><td>For steps 5-9, prepare an appropriate volume of working reagents.
Plan for 30 µL/well for 96-well and 15 µL/well for 384-well plates and include overage to accommodate automation equipment dead volumes.
Keep working reagents at room temperature until use. Working reagents are good for 2 days. If unused on the day of preparation, store at 4 °C overnight and rewarm to 40 °C for 30 minutes before use.</td></tr>
<tr><td>5</td><td>Prepare Protease Working Solution. Dilute Protease 1:4,000 (or concentration optimized for your cell type) with room temperature 1X PBS. Vortex briefly to mix.
IMPORTANT Optimal Protease dilution needs to be determined. Refer to “Appendix IV: Optimizing Assay Conditions for a New Cell Type” on page 25 for more information.</td></tr>
<tr><td>6</td><td>Prepare Working Probe Set Mixture. Dilute TYPE 1 and TYPE 2 Probe Sets 1:50 in 40 °C, prewarmed Hyb A Buffer. Vortex briefly to mix.
! WARNING ! Hyb A Buffer contains formamide, a teratogen, irritant, and possible carcinogen. Avoid contact with skin and mucous membranes.
<table>
<tr><th>Component</th><th>µL Per Well (384-well plate)</th><th>µL Per Well (96-well plate)</th></tr>
<tr><td>Hyb A (prewarmed)</td><td>14.4</td><td>28.8</td></tr>
<tr><td>TYPE 1 Probe Set</td><td>0.3</td><td>0.6</td></tr>
<tr><td>TYPE 2 Probe Set</td><td>0.3</td><td>0.6</td></tr>
<tr><td>Total volume</td><td>15</td><td>30</td></tr>
</table></td></tr>
<tr><td>7</td><td>Prepare Working PreAmp Mixture. Dilute PreAmp (or PreAmps) 1:250 in 40 °C, prewarmed Hyb B Buffer. Vortex briefly to mix.
! WARNING ! Hyb B Buffer contains formamide, a teratogen, irritant, and possible carcinogen. Avoid contact with skin and mucous membranes.
<table>
<tr><th>Component</th><th>µL Per Well (384-well plate)</th><th>µL Per Well (96-well plate)</th></tr>
<tr><td>Hyb B (prewarmed)</td><td>14.88</td><td>29.76</td></tr>
<tr><td>PreAmp 1</td><td>0.06</td><td>0.12</td></tr>
<tr><td>PreAmp 2</td><td>0.06</td><td>0.12</td></tr>
<tr><td>Total volume</td><td>15</td><td>30</td></tr>
</table></td></tr>
</table>

To prepare reagents: *(continued)*

Step	Action
8	Prepare Working Amp Mixture. Dilute Amp (or Amps) 1:250 in 40 °C, prewarmed Hyb B Buffer. Vortex briefly to mix.

Component	µL Per Well (384-well plate)	µL Per Well (96-well plate)
Hyb B (prewarmed)	14.88	29.76
Amp 1	0.06	0.12
Amp 2	0.06	0.12
Total volume	15	30

Step	Action
9	Prepare Working Label Probe Mixture. Dilute LP (or LPs) 1:250 in 40 °C, prewarmed Hyb C Buffer. Vortex briefly to mix.

Component	µL Per Well (384-well plate)	µL Per Well (96-well plate)
Hyb C (prewarmed)	14.88	29.76
LP 1	0.06	0.12
LP 2	0.06	0.12
Total volume	15	30

Fixing Cells and Treating with Protease

To fix and treat cells:

Step	Action
1	Fix cells in Formaldehyde Solution: **! WARNING ! Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.** a. Aspirate medium from cells leaving defined residual volume and gently wash two times with 1X PBS. Refer to the table below for volumes. b. Aspirate the final 1X PBS wash, dispense 8% Formaldehyde Solution, and incubate for 30 minutes at room temperature. c. Aspirate the Formaldehyde Solution and gently wash three times with 1X PBS.

Per Well	Residual Vol (µL)	1X PBS Vol (µL)	8% Formaldehyde Solution Vol (µL)
96-well plate	30	150	30
384-well plate	15	80	15

Note Optional. At this point, the assay can be stopped by dehydration and storage at –20 °C. See “Appendix II: Procedure for Dehydrating Cells for Storage at –20 °C, or for Shipping” on page 23 for a procedure.

To fix and treat cells: *(continued)*

Step	Action
2	Permealize cells with Detergent Solution: a. Dispense Detergent Solution and incubate for 3 minutes at room temperature. b. Aspirate Detergent Solution and wash twice with 1X PBS.

Per Well	Residual Vol (µL)	Detergent Vol (µL)	1X PBS Vol (µL)
96-well plate	30	30	150
384-well plate	15	15	80

Step	Action
3	Digest with Protease: **IMPORTANT** The Protease dilution used in this example should be sufficient for a majority of cell lines. If you observe high cell loss or no signal, the optimal dilution needs to be determined. Refer to "Appendix IV: Optimizing Assay Conditions for a New Cell Type" on page 25 for more information. a. Aspirate the 1X PBS from the plate, dispense Protease Working Solution, cover plate with plate lid, and incubate for 10 minutes at room temperature. b. Aspirate the Protease solution and gently wash 5 times with 1X PBS. c. Aspirate 1X PBS, dispense Protease Stop Buffer, and pipet up and down twice to mix. d. Aspirate Protease Stop Buffer and dispense fresh Protease Stop Buffer. e. Proceed to hybridization procedure. Samples may sit in Protease Stop Buffer for up to 30 minutes.

Per Well	Residual Vol (µL)	Diluted Protease Vol (µL)	1X PBS Vol (µL)	Protease Stop Buffer Vol (µL)
96-well plate	30	30	150	30
384-well plate	15	15	80	15

Performing Hybridizations

To perform hybridizations:

Step	Action
1	Hybridize target Probe Sets: a. Aspirate Protease Stop Buffer and dispense Working Probe Set Mixture. b. Cover plate with plate lid and incubate plate for 3 hours at 40 °C +/- 1 °C.

Per Well	Residual Vol (µL)	Working Probe Set Vol (µL)
96-well plate	30	30
384-well plate	15	15

To perform hybridizations: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>2</td><td>Aspirate the Working Probe Set Mixture and wash 3 times with Wash Buffer. Include a 30 second soak at the last wash step.
<table>
<tr><th>Per Well</th><th>Residual Vol (µL)</th><th>Wash Buffer Vol (µL)</th></tr>
<tr><td>96-well plate</td><td>30</td><td>150</td></tr>
<tr><td>384-well plate</td><td>15</td><td>80</td></tr>
</table></td></tr>
<tr><td>3</td><td>Optional. Plates can be stored at 4 °C for up to 24 hours. To continue without storage, proceed to step 4.

Prepare for storage:
a. Aspirate Wash Buffer and dispense Storage Buffer.
b. After storage, aspirate Storage Buffer and wash the plate twice with Wash Buffer.

IMPORTANT Ensure that the refrigeration is at 4 °C. Lower temperatures will cause precipitation of Storage Buffer resulting in significant cell loss.
<table>
<tr><th>Per Well</th><th>Residual Vol (µL)</th><th>Storage Buffer Vol (µL)</th><th>Wash Buffer Vol (µL)</th></tr>
<tr><td>96-well plate</td><td>30</td><td>30</td><td>150</td></tr>
<tr><td>384-well plate</td><td>15</td><td>15</td><td>80</td></tr>
</table></td></tr>
<tr><td>4</td><td>Hybridize PreAmplifiers (PreAMPs):

If continuing from step 3, make sure working reagents stored at 4 °C have been prewarmed and are ready (see “Preparing Reagents” on page 10).
a. Aspirate Wash Buffer, dispense PreHyb Buffer prewarmed to 40 °C, and pipet up and down twice to mix.
b. Aspirate PreHyb Buffer and dispense Working PreAMP Mixture.
c. Cover plate with plate lid and incubate at 40 °C +/- 1 °C for 60 minutes.
<table>
<tr><th>Per Well</th><th>Residual Vol (µL)</th><th>PreHyb Buffer Vol (µL)</th><th>Working PreAmp Vol (µL)</th></tr>
<tr><td>96-well plate</td><td>30</td><td>30</td><td>30</td></tr>
<tr><td>384-well plate</td><td>15</td><td>15</td><td>15</td></tr>
</table></td></tr>
<tr><td>5</td><td>Aspirate the Working PreAMP Mixture and wash 3 times with Wash Buffer. Include a 30 second soak at the last wash step.
<table>
<tr><th>Per Well</th><th>Residual Vol (µL)</th><th>Wash Buffer Vol (µL)</th></tr>
<tr><td>96-well plate</td><td>30</td><td>150</td></tr>
<tr><td>384-well plate</td><td>15</td><td>80</td></tr>
</table></td></tr>
</table>

To perform hybridizations: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>6</td><td>Hybridize Amplifiers (AMPs):
a. Aspirate Wash Buffer, dispense PreHyb Buffer prewarmed to 40 °C, and pipet up and down twice to mix.
b. Aspirate PreHyb Buffer and dispense Working AMP Mixture.
c. Cover plate with plate lid and incubate at 40 ± 1 °C for 60 minutes.

<table><tr><th>Per Well</th><th>Residual Vol (µL)</th><th>PreHyb Buffer Vol (µL)</th><th>Working Amp Vol (µL)</th></tr><tr><td>96-well plate</td><td>30</td><td>30</td><td>30</td></tr><tr><td>384-well plate</td><td>15</td><td>15</td><td>15</td></tr></table></td></tr>
<tr><td>7</td><td>Aspirate the Working AMP Mixture and wash 3 times with Wash Buffer. Include a 30 seconds soak at the last wash step.

<table><tr><th>Per Well</th><th>Residual Vol (µL)</th><th>Wash Buffer Vol (µL)</th></tr><tr><td>96-well plate</td><td>30</td><td>150</td></tr><tr><td>384-well plate</td><td>15</td><td>80</td></tr></table></td></tr>
<tr><td>8</td><td>IMPORTANT Protect samples from light during this and all subsequent steps.

Hybridize Label Probes (LPs):
a. Dispense PreHyb Buffer prewarmed to 40 °C, and pipet up and down twice to mix.
b. Aspirate PreHyb Buffer and dispense Working LP Mixture.
c. Cover plate with plate lid and incubate at 40 ± 1 °C for 60 minutes.

<table><tr><th>Per Well</th><th>Residual Vol (µL)</th><th>PreHyb Buffer Vol (µL)</th><th>Working LP Vol (µL)</th></tr><tr><td>96-well plate</td><td>30</td><td>30</td><td>30</td></tr><tr><td>384-well plate</td><td>15</td><td>15</td><td>15</td></tr></table></td></tr>
<tr><td>9</td><td>Wash three times with Wash Buffer:
a. Aspirate the Working LP Mixture and wash 3 times with Wash Buffer. Include a 30 second soak at the last wash step.
b. Dispense 1X PBS. At this point, the plate is stable at room temperature for several hours.

<table><tr><th>Per Well</th><th>Residual Vol (µL)</th><th>Wash Buffer Vol (µL)</th><th>1X PBS Vol (µL)</th></tr><tr><td>96-well plate</td><td>30</td><td>150</td><td>150</td></tr><tr><td>384-well plate</td><td>15</td><td>80</td><td>80</td></tr></table></td></tr>
</table>

To perform hybridizations: *(continued)*

Step	Action
10	Counterstain nuclei with DAPI solution: **! WARNING ! DAPI is a possible mutagen. Avoid contact with skin and mucous membranes.** **IMPORTANT** Protect from light. a. Prepare DAPI Working Solution by diluting DAPI Stock Solution (10 mg/mL) 1:5,000 in 1X PBS and briefly vortexing to mix well. For example, 1 µL DAPI stock in 5 mL of 1X PBS. Scale reagents as appropriate (see below).

Component	Vol (µL) Per Well 384-Well Plate	Vol (µL) Per Well 96-Well Plate
1X PBS	14.997	29.994
10 mg/mL DAPI	0.003	0.006
Total Volume	**15**	**30**

b. Aspirate 1X PBS and dispense DAPI Working Solution.
c. Incubate at room temperature for 1 minute.
d. Aspirate the DAPI Working Solution and wash once with 1X PBS.
e. Aspirate the 1X PBS and dispense fresh 1X PBS.

The plate is ready for imaging. Alternatively, it can be stored at 4 °C for several days. Seal the plate with an adhesive seal when storing at 4 °C.

Note Signals drop by approximately 50% after 2 days.

Per Well	Residual Vol (µL)	DAPI (µL)	1X PBS (µL)	1X PBS Final (µL)
96-well plate	30	30	150	150
384-well plate	15	15	80	80

To perform hybridizations: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>11</td><td>Scan the plate on an imaging system by:
♦ Using DAPI, fluorescein and rhodamine channels, or by
♦ Visualizing under a fluorescent microscope using appropriate filter sets
We recommend the following:
♦ Magnify the image by 200-400-fold through the combined use of a 10X eyepiece and a 20-40X fluorescence objective with numeric aperture equal to or greater than 0.75
♦ Set exposure time to obtain optimal signal-to-background ratio. Normally, DAPI stain requires 10-50X less exposure time than for target RNA 1 and 2.
♦ Use the following table to determine the multi-bandpass fluorescence microscope filter sets to visualize signals. For the best viewing signal, set the gray scale or intensity level as follows:
– min = 2X above background outside of cell
– max = 4X above background outside of cell
– Adjust as necessary

<table>
<tr><th>Nucleic Acid</th><th>Fluorophore</th><th>Peak Excitation (nm)</th><th>Peak Emission (nm)</th><th>Purpose</th></tr>
<tr><td>Target RNA 1</td><td>Fluorescein</td><td>495</td><td>519</td><td>For detection of 1 copy/cell</td></tr>
<tr><td>Target RNA 2</td><td>Rhodamine</td><td>546</td><td>573</td><td>For detection of greater than 1000 copies/cell</td></tr>
<tr><td>Nuclear DNA</td><td>DAPI</td><td>358</td><td>461</td><td>For focusing and cell identification</td></tr>
</table>
</td></tr>
</table>

Section V: Troubleshooting

Troubleshooting No or Weak Signals

Troubleshooting no or weak signals:

Probable Cause	Recommended Actions
Inadequate Protease digestion	Optimize Protease digestion conditions as described in “Appendix IV: Optimizing Assay Conditions for a New Cell Type” on page 25.
Over-fixing cells	Do not exceed the recommended fixation time for your cell type.
Over/under permeabilization	Perform time titration of permeabilization step for 1-10 minutes.
Inappropriate hybridization temperature	Hybridization reactions must be carried out at 40 °C ± 1 °C. Use a QuantiGene Incubator Temperature Validation Kit to verify and monitor the temperature.
Incorrect Use of Probe Sets, PreAMP, AMP and/or LP	Ensure that Working Solutions are prepared properly and used in the correct order. If running singleplex assays, use Type 1 and 2 Probe Sets with PreAMP/AMP/LP 1 and 2, respectively. If running multiplex assays, include Type 1 and 2 Probe Sets and PreAMP/AMP/LP 1 and 2 in the appropriate Working Solutions.
Photo-bleaching of fluorescent signals	Cover samples with foil during LP hybridization and all subsequent steps. Store samples in the dark.
Inappropriate microscope set-up or operation	Ensure that your microscope is in good working order and that your light source, objectives, filters, and exposure time are selected properly.
Poor cell retention	Titrate cell numbers, fixation, and Protease treatments. Try using different plate type to ensure good cell adhesion. Plastic plates work better but tend to exhibit 2-4X higher background. Try coating the plating surfaces with different extracellular matrices such as MatriGel or Poly-D-lysine. Use 5.0 µL/sec dispensing/aspiration speed to minimize cell detachment. Dispense and aspirate near the edge of the well and image on the opposite edge of the wells. Use residual volume protocol. If necessary, increase amount of residual volume. The volume of all reagents in all steps must be increased proportionally. For example, if there is 60 µL of residual volume, then 60 µL of reagents must be used. Use adherent cells and ensure cells are firmly adhered to the plate prior to the assay.

Variable Staining Within a Sample

Troubleshooting variable staining within a sample:

Probable Cause	Recommended Actions
Samples were allowed to dry	Once the samples have been rehydrated, do not allow the cells to dry at any stage. Do not use less than the recommended volumes of any hybridization solution for your sample type. Use residual volume method or leave recommended volume after each wash step. This will minimize the chance of a well drying out completely.
Non-uniform aspiration/dispensing of the multi-channel pipettor	Correct and verify uniform volume for aspiration and dispense of all pipet tips.
Insufficient mixing of reagents	Pre-warm hybridization buffers to re-dissolve any precipitates before use. Keep PreHyb Buffer at 40 °C at all times before adding to the plate. Briefly vortex all working hybridization solutions to mix well before use.
Located outside depth of field of objective lens	Adjust focus, avoid using non-uniform and thick plastic plates.

High Background on Entire Plates

Troubleshooting high background on plates:

Probable Cause	Recommended Actions
Samples were allowed to dry	Once the in situ part of the assay is started, do not allow the cells to dry at any stage. Do not use less than the recommended volumes of any hybridization solution for your sample type. Leaving recommended residual volume after each wash step will minimize the chance of a well drying completely.
Insufficient washing	Make sure you wash according to the procedure.
High autofluorescence from the plates or extracellular matrices	Select plates or extracellular matrix that exhibit no or low autofluorescence. Test background with cells only and without Probe Set, PreAmp, Amp, and Label Probe.
Incorrect filter setup	Replace filters used in the imaging system.

High Background Within Cells

Troubleshooting high background within cells:

Probable Cause	Recommended Actions
Samples were allowed to dry	Once the in situ part of the assay is started, do not allow the cells to dry at any stage. Do not use less than the recommended volumes of any hybridization solution for your sample type. Use residual volume method or leave recommended volume after each wash step. This will minimize the chance of a well drying out completely.
Insufficient washing	Add an additional wash step to all washes.
Non-specific binding of Probe Set(s)	Hybridization reactions must be carried out at 40 °C ± 1 °C. Use a QuantiGene Incubator Temperature Validation Kit to verify and monitor the temperature.
Insufficient Protease digestion	Optimize Protease digestion conditions as described in “Appendix IV: Optimizing Assay Conditions for a New Cell Type” on page 25.

High Number of Dots Appear where Cells are Absent

Troubleshooting a significant number of dots outside the cells:

Probable Cause	Recommended Actions
Non-specific binding of Probe Set, PreAmp, Amp, or Label Probe to plate	Verify by running the assay without cells in wells.
High cell loss and cell damage from excess detergent, Protease treatment, or fixation	Optimize detergent, Protease, and fixation conditions.

No DAPI or Dots

Troubleshooting imaging problems:

Probable Cause	Recommended Actions
Incorrect filter setup	Verify that the correct filters are installed. See the Technical Note “Verification of microscope suitability for ViewRNA”.
Plate and imaging system incompatible	Verify by scanning for nuclear DAPI stain. The signal should be strong and sharp. If it is not, switch to a plate type that is suitable for the imaging system or adjust the imaging to match the plate type. This involves the adjustment of the Z-axis.

Appendix I: Liquid Handling Recommendations

Fluidic Handling Systems

Pipetting Stations

- Platemate 2x3
- Biomek FX or NX
- Velocity11 Bravo

Plate Washers

- BioTek ELx405 Select

Pipetting Station and Plate Washer Combination

- BioTek EL406

Minimum Requirements

- 96- or 384-channel pipette head
- 80 µL +/- 5% pipette capacity (30 µL is acceptable but will result in increased processing time)
- Tips can be replaced or washed between dispensings

Setup Recommendations

The goal of these recommendations is to minimize cell loss.

- Start with a dispense/aspirate rate to 5 µL/sec. Slowly increase the flow rate, making sure that cells are not detaching in the process. If the liquid handling is setup properly, 80% of the cells will remain following the procedure. If you use the recommended residual volume protocol, dispense/aspirate rates of 20 µL/sec could be achieved.
- Set a residual volume to remain after each aspiration, 15 µl/well for 384-well plates and 30 µL/well for 96-well plates. This amount of residual liquid is accounted for in the assay procedures and will minimize cell detachment. If you must increase the residual volume, adjust the quantities of the assay reagents proportionally so that they match the residual volume.
- Orient the pipet tip toward the left or right side of the well and then capture the image on the opposite side. Less cell loss should be observed on the opposite side of the pipet tip location.

Wash Protocol Settings for BioTek ELx405 Select

IMPORTANT Settings might require some adjustment since different instruments of the same model can behave differently.

Method	Setting
Number of Cycles	Per assay protocol
Wash format	Plate
Soak/Shake	Per assay protocol

Aspirate	384-Well Plate	96-Well Plate
Asp height	027 (3.429 mm)	036 (4.572 mm)
Horizontal asp pos	-25 (-1.143 mm)	-45 (-2.057 mm)
Horiz y asp pos	00 (00.00 mm)	00

Aspiration rate	01 (3.0 mm/sec)	1 (3.0 mm/sec)
Aspiration delay	0000 (msec)	0
Crosswise aspiration	No	No
Final aspiration	Yes	Yes
Final aspiration delay	0000 (msec)	0
Dispense	**384-Well Plate**	**96-Well Plate**
Dispense volume	80 µL/well	150 µL/well
Dispense flow rate	01	01
Dispense height	120 (15.240 mm)	120
Horizontal disp pos	-15 (-0.686 mm)	-35 (-1.600 mm)
Horiz Y disp pos	00 (0.000 mm)	00
Bottom wash first	No	No
Prime by start	No	No

Appendix II: Procedure for Dehydrating Cells for Storage at –20 °C, or for Shipping

About Dehydration Procedure

Use this procedure if you want to temporarily stop the assay after fixing the cells. Following dehydration, store the cells at –20 °C for up to 2 months or until ready to use. When ready to use, follow the procedure for rehydrating the cells before continuing with the permeabilization step.

IMPORTANT Following the dehydration-rehydration steps, plates with Matrigel (but not poly-a-lysine or collagen) coatings might have a significant increase in background. Verify that this will not be an issue before preparation and storage of multiple plates.

Procedure

The 50 µL/well volume in this procedure is specific for 384-well plates. If you are using 96-well plates, increase the volume from 50 µL/well to 100 µL/well.

IMPORTANT Do not apply the residual volume technique to this procedure.

To dehydrate cells for storage or for shipping:

Step	Action
1	To dehydrate cells: a. Aspirate the final 1X PBS wash, then dispense 50 µL/well 50% ethanol. b. Incubate at room temperature (RT) for 2 minutes. c. Aspirate 50% ethanol, then dispense 50 µL/well 70% ethanol. d. Incubate at RT for 2 minutes. e. Aspirate 70% ethanol, then dispense 50 µL/well 100% ethanol. f. Aspirate 100% ethanol, then dispense 50 µL/well fresh 100% ethanol. g. Cover the plate with its lid and seal with parafilm. h. Store at –20 °C. Cells can be stored for several weeks. Proceed to step 3 when you are ready to run the rest of the assay. i. Proceed to step 2 if you plan to ship the plate. **IMPORTANT** Glass bottom plates may crack when stored at –20 °C. In this case, store at 4 °C for up to 48 hours.
2	To prepare for shipping: a. Aspirate the ethanol. b. Seal the plate with an adhesive seal. c. Ship the plate overnight at 4 °C. d. Immediately upon receipt of the plate, add 100% ethanol and store at –20 °C.
3	When ready to run the rest of the assay, rehydrate the cells: a. Aspirate 100% ethanol, and dispense 50 µL/well 70% ethanol. b. Incubate at RT for 2 minutes. c. Aspirate 70% ethanol, and dispense 50 µL/well 50% ethanol. d. Incubate at RT for 2 minutes. e. Aspirate 50% ethanol and dispense 50 µL/well 1X PBS. f. Incubate at RT for 10 minutes. Proceed to step 2 on page 13, and continue with the permeabilization of the cells.

Appendix III: 96- and 384-Well Imaging-Compatible Plates

Compatible Plates

Below is a table of plates that have been shown to work with our assay. Please refer to “Selecting the Appropriate Plate type” on page 6 for criteria to consider when selecting a plate.

Format	Vendor	Cat #	Bottom	Coating
384-well	Greiner	781946	Polystyrene	Poly-D-lysine
	MatriCal	MGB101-1-2-LG-L	Glass	None
	Perkin Elmer CellCarrier/TC 384	6007439	Polystyrene	None
96-well	Nunc	165305	Polymer	None
	Nunc	164588	Glass	None
	BD Biocoat	35-4640 or 35-6640	Polystyrene	Poly-D-lysine

Appendix IV: Optimizing Assay Conditions for a New Cell Type

Initial Optimization and Assessment of Assay Backgrounds

In the initial experiment, we recommend you start with a single plex assay, 80-95% cell density, 30 minute fixation time, and a 10 minute incubation time with 4 different Protease dilutions. Refer to “Assessing Background for New Projects” on page 6 for details on recommended controls for assessing backgrounds. The table below is a recommended plate map for an initial optimization assay.

Sample plate map for a 384-well plate:

Row	Control Type	Protease Dilution			
A-B	No cells	**1:1,000 for well columns 1-6**	**1:2,000 for well columns 7-12**	**1:4,000 for well columns 13-18**	**1:8,000 for well columns19-24**
C-D	Cells only				
E-F	No Probe Set				
G-H	Negative control Probe Set				
I-J	Positive control Probe Set				
K-L	New TYPE 1 Probe Set only				
M-N	New TYPE 2 Probe Set only				
O-P	Both TYPE 1 and 2 Probe Sets				

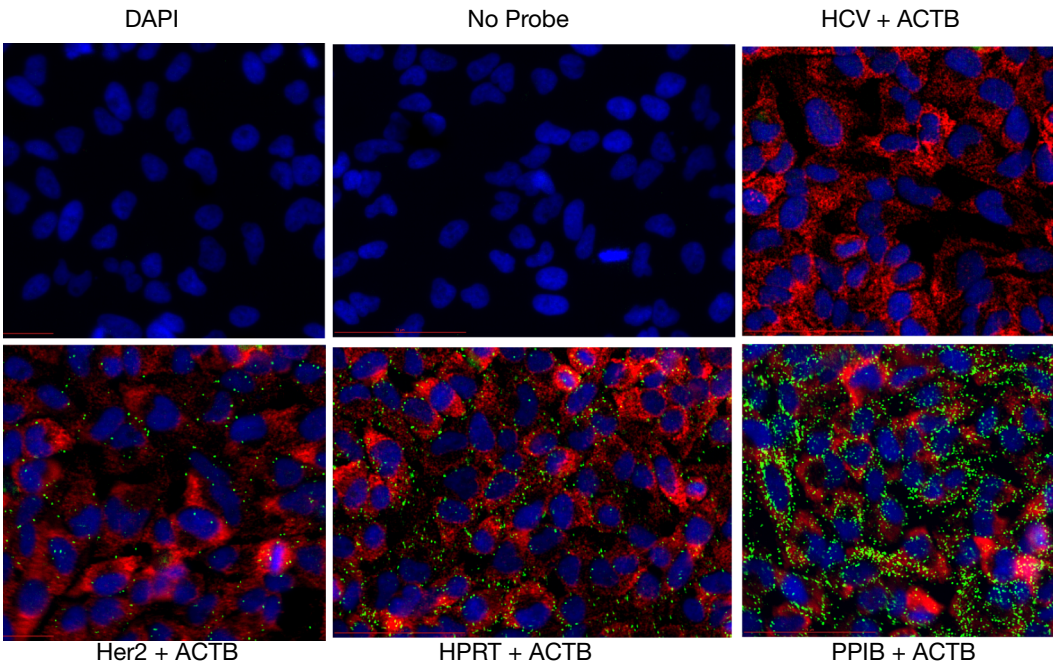
From each optimization experiment, select the optimal conditions based on the following criteria:

- Least cell loss
- Lowest background inside and outside cells in the negative control wells (no dots and low, uniform background)
- Highest signal/background ratio of dots inside the cell
- Best assay precision between replicate wells and plates for cell number, low background, and high signal/background ratio
- When running both a TYPE 1 and TYPE 2 Probe Set, weight your selection for optimal assay conditions for the TYPE 1 Probe Set

In some cases, finding the optimal conditions may require an additional experiment. See the following section for a description of a plate map for further optimization.

Examples of Optimized Conditions

The images below show representative examples of optimized assay conditions.



HeLa cells were cultured, fixed, permeabilized, and protease treated in a 96-well clear bottom, black-wall plate. The cells were then probed using a TYPE 2 Probe Set for ACTB (red color) and TYPE 1 Probe Sets for HCV, Her2, HPRT, and PPIB (green color). After QuantiGene ViewRNA branched DNA amplification and DAPI nuclear stain, the HeLa cells in the plate were scanned on a CellWoRx scanner using the 20X objective lens. The exposure time and the intensity settings used for each channel are summarized below.

Typical results of HeLa positive control. No signal is observed in the negative controls (DAPI and No Probe). Green, but not red signal can be observed in the panel of HCV + ACTB. Increasing number of green dots are observed in panels representing expression of Her2, HPRT, and PPIB, respectively, whereas ACTB signal remains the same.

Channel	Exposure Time (seconds)	Min/Max Intensity Setting
DAPI	0.2	4,000/50,000
Fluorescein	3	750/2,000
Rhodamine	3	1,000/10,000

Additional Optimization

If acceptable data is not obtained during the initial optimization assay, it may be necessary to evaluate additional cell densities and/or fixation times. A recommended plate map is provided below.

Sample plate map for a 384-well plate:

Row	Fixation Time (min)	Control Type	Protease Dilution			
			1:1,000 for well columns 1-6	**1:2,000 for well columns 7-12**	**1:4,000 for well columns 13-18**	**1:8,000 for well columns 19-24**
A	15	No Probe Set				
B		Positive control Probe Set				
C		New TYPE 1 Probe Set only				
D		New TYPE 2 Probe Set only				
E		Both TYPE 1 and 2 Probe Sets				
F	30	No Probe Set				
G		Positive control Probe Set				
H		New TYPE 1 Probe Set only				
I		New TYPE 2 Probe Set only				
J		Both TYPE 1 and 2 Probe Sets				
K	45	No Probe Set				
L		Positive control Probe Set				
M		New TYPE 1 Probe Set only				
N		New TYPE 2 Probe Set only				
O		Both TYPE 1 and 2 Probe Sets				

Appendix V: Alternative Assay Procedure (Manual Processing)

Overview

The following procedures can be completed in one long day, or broken down between 2 days. Cells must be plated in 96-well format and allowed to adhere at least 1 day before starting the Assay Procedure.

IMPORTANT This alternative procedure is optimized for using multichannel pipettor for manual microplate manipulation.

IMPORTANT Before starting this procedure, carefully review "Section III: Recommendations for Experimental Design and Assay Optimization" on page 6.

Assay Workflow

- Prepare cells by fixing, permeabilizing, and digesting with Protease
- Perform hybridizations
- Analyze plate

Important Procedural Notes

- Hybridization reactions must be carried out at 40 ±1 °C. Verify and monitor oven temperature using the QuantiGene Incubator Temperature Validation Kit.
- Protect samples from light during the Label Probe hybridization and all subsequent steps.
- Before opening reagents supplied in microfuge tubes, briefly centrifuge to collect contents at the bottom of the tube.
- All Hyb Buffers stored at 4 °C must be prewarmed to 40 °C for 30 minutes to redissolve any precipitates. Precipitation will form at room temperature.

Preparing Reagents

To prepare reagents for alternative processing procedure:

Step	Action
1	Thaw/warm the following reagents: ♦ Prewarm PreHyb, Hyb A, Hyb B, and Hyb C Buffers to 40 °C for 30 minutes ♦ Thaw Probe Sets, PreAmp (or PreAmps), Amp (pr Amps), and LP (or LPs) and place on ice
2	Prepare the appropriate volume of the following reagents: ♦ 1X PBS. 250 mL is sufficient for one 96-well plate. ♦ 4% Formaldehyde Solution in 1X PBS (prepare daily). 7.2 mL is sufficient for one 96-well plate. **! WARNING ! Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.**
3	Prepare Wash Buffer. For example, to a 1,000-mL graduated cylinder, add the following in this order: ♦ 800 mL nuclease-free water ♦ 3 mL Wash Buffer Comp 1 ♦ 5 mL Wash Buffer Comp 2 ♦ Adjust to 1 liter with nuclease-free water Scale preparation according to the number of plates to be processed. 200 mL is sufficient for processing one 96-well plate.

To prepare reagents for alternative processing procedure: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>4</td><td>For steps 5-16, prepare an appropriate volume of working reagents.

Plan for 60 µL/well for 96-well plates and include overage to accommodate the use of reagent reservoirs. Volumes sufficient for one 96-well plate are provided throughout the procedures.

Keep working reagents at room temperature until use. Working reagents are good for 2 days. If unused the day of preparation, store at 4 °C overnight and rewarm to 40 °C for 30 minutes before use. Swirl working reagents to visually verify there are no precipitates.</td></tr>
<tr><td>5</td><td>Prepare Working Detergent Solution by diluting stock Detergent Solution 1:1 with 1X PBS and then vortexing briefly to mix.

8 mL is sufficient for one 96-well plate.</td></tr>
<tr><td>6</td><td>Prepare Working Protease Solution by diluting Protease 1:8,000 (or optimized concentration for your cell type) with room temperature 1X PBS and then vortexing briefly to mix.

8 mL is sufficient for one 96-well plate.</td></tr>
<tr><td>7</td><td>Prepare Working Protease Stop Buffer by diluting stock Protease Stop Buffer 3:1 with 1X PBS and then vortexing briefly to mix.

Prepare enough for the assay and also for the preparation Working Hyb A Buffer.

13 mL (9.75 mL stock Protease Stop Buffer plus 3.25 mL 1X PBS) is sufficient for one 96-well plate.</td></tr>
<tr><td>8</td><td>Prepare Working Hyb A Buffer by diluting stock Hyb A Buffer, prewarmed to 40 °C, 1:1 with Working Protease Stop Buffer and then vortexing briefly to mix.

! WARNING ! Hyb A Buffer contains formamide, a teratogen, irritant, and possible carcinogen. Avoid contact with skin and mucous membranes.

8 mL is sufficient for one 96-well plate.</td></tr>
<tr><td>9</td><td>Prepare Working Probe Set Mixture by diluting TYPE 1 and TYPE 2 Probe Sets 1:100 with Working Hyb A Buffer and then vortexing briefly to mix.

8 mL is sufficient for one 96-well plate.

<table>
<tr><th>Component</th><th>Vol (µL) Per Well (96-well plate)</th></tr>
<tr><td>Working Hyb A (prewarmed)</td><td>58.8</td></tr>
<tr><td>TYPE 1 Probe Set</td><td>0.6</td></tr>
<tr><td>TYPE 2 Probe Set</td><td>0.6</td></tr>
<tr><td>Total volume</td><td>60</td></tr>
</table>
</td></tr>
<tr><td>10</td><td>Prepare Working Storage Buffer by diluting stock Storage Buffer 1:1 with nuclease-free water and then vortexing briefly to mix.

8 mL is sufficient for one 96-well plate.</td></tr>
<tr><td>11</td><td>Prepare Working PreHyb Buffer by diluting stock PreHyb Buffer, prewarmed to 40 °C, 1:1 with nuclease-free water and then vortexing briefly to mix.

Prepare enough for the preparation of Working Hyb B and C Buffers.

13 mL is sufficient for one 96-well plate.</td></tr>
</table>

To prepare reagents for alternative processing procedure: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>12</td><td>Prepare Working Hyb B Buffer by diluting stock Hyb B Buffer, prewarmed to 40 °C, 1:1 with Working PreHyb Buffer and then vortexing briefly to mix.

! WARNING ! Hyb B Buffer contains formamide, a teratogen, irritant, and possible carcinogen. Avoid contact with skin and mucous membranes.

Prepare enough for the preparation of Working PreAmp Mixture and Working Amp Mixture.

16 mL is sufficient for one 96-well plate.</td></tr>
<tr><td>13</td><td>Prepare Working Hyb C Buffer by diluting stock Hyb C Buffer, prewarmed to 40 °C, 1:1 with Working PreHyb Buffer and then vortexing briefly to mix.

8 mL is sufficient for one 96-well plate.</td></tr>
<tr><td>14</td><td>Prepare Working PreAmp Mixture by diluting PreAmp (or PreAmps) 1:500 with Working Hyb B Buffer and then vortexing briefly to mix.

8 mL is sufficient for one 96-well plate.

<table>
<tr><th>Component</th><th>Vol (µL) Per Well (96-well plate)</th></tr>
<tr><td>Working Hyb B</td><td>59.76</td></tr>
<tr><td>PreAmp 1</td><td>0.12</td></tr>
<tr><td>PreAmp 2</td><td>0.12</td></tr>
<tr><td>Total volume</td><td>60</td></tr>
</table>
</td></tr>
<tr><td>15</td><td>Prepare Working Amp Mixture by diluting Amp (or Amps) 1:500 with Working Hyb B Buffer and then vortexing briefly to mix.

8 mL is sufficient for one 96-well plate.

<table>
<tr><th>Component</th><th>Vol (µL) Per Well (96-well plate)</th></tr>
<tr><td>Working Hyb B</td><td>59.76</td></tr>
<tr><td>Amp 1</td><td>0.12</td></tr>
<tr><td>Amp 2</td><td>0.12</td></tr>
<tr><td>Total volume</td><td>60</td></tr>
</table>
</td></tr>
<tr><td>16</td><td>Prepare Working Label Probe Mixture by diluting LP (or LPs) 1:500 with Working Hyb C Buffer and then vortexing briefly to mix.

8 mL is sufficient for one 96-well plate.

<table>
<tr><th>Component</th><th>Vol (µL) Per Well (96-well plate)</th></tr>
<tr><td>Working Hyb C</td><td>59.76</td></tr>
<tr><td>LP 1</td><td>0.12</td></tr>
<tr><td>LP 2</td><td>0.12</td></tr>
<tr><td>Total volume</td><td>60</td></tr>
</table>
</td></tr>
</table>

Fixing Cells and Treating with Protease

To fix and treat cells:

Step	Action
1	Fix cells in 4% Formaldehyde Solution: **! WARNING ! Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.** a. Invert plate containing cells over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. Gently wash 2 times with 150 µL/well 1X PBS. b. Remove (as above) the final 1X PBS wash, add 60 µL 4% Formaldehyde Solution, cover plate with plate lid and incubate for 30 minutes at room temperature. c. Remove (as above) the Formaldehyde Solution and gently wash 3 times with 150 µL/well 1X PBS. **Note** Optional. At this point, the assay can be stopped by dehydration and storage at –20 °C. See “Appendix II: Procedure for Dehydrating Cells for Storage at –20 °C, or for Shipping” on page 23 for a procedure.
2	Permealize cells with Working Detergent Solution: a. Add 60 µL/well Working Detergent Solution and incubate for 3 minutes at room temperature. b. Invert plate over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. c. Add 150 µL/well 1X PBS.
3	Digest with Working Protease Solution: **IMPORTANT** The Protease dilution used in this example should be sufficient for a majority of cell lines. If you observe high cell loss or no signal, the optimal dilution should be determined. Refer to “Appendix IV: Optimizing Assay Conditions for a New Cell Type” on page 25 for more information. a. Invert plate over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. Add 60 µL/well Working Protease Solution, cover plate with plate lid, and incubate for 10 minutes at room temperature. b. Remove (as above) the Working Protease Solution and gently wash 3 times with 150 µL/well 1X PBS. c. Remove (as above) 1X PBS and add 60 µL/well Working Protease Stop Buffer. d. Proceed to hybridization procedure. Samples may sit in Working Protease Stop Buffer for up to 30 minutes.

Performing Hybridizations

To perform hybridizations:

Step	Action
1	Hybridize target Probe Sets: a. Invert plate over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. Add 60 µL/well Working Probe Set Mixture. b. Cover plate with plate lid and incubate plate for 3 hours at 40 °C +/- 1 °C.

To perform hybridizations:

Step	Action
2	Invert plate over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. Wash 3 times with 150 µL/well Wash Buffer.
3	*Optional.* Plates can be stored at 4 °C for up to 24 hours. To continue without storage, proceed to step 4. Prepare for storage: a. Invert plate over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. Add 60 µL/well Working Storage Buffer. b. Plate is now ready for storage. Cover with plate lid, wrap with parafilm, and store at 4 °C. c. After storage, remove (as above) Working Storage Buffer and wash the plate 2 times with 150 µL/well Wash Buffer before hybridizing to the preamplifier. **IMPORTANT** Ensure that the refrigeration is at 4 °C. Lower temperatures will cause precipitation of Storage Buffer resulting in significant cell loss.
4	Hybridize Pre-Amplifiers (PreAMPs): If continuing from step 3 following storage, make sure working reagents stored at 4 °C have been prewarmed and are ready. See “Preparing Reagents” on page 28. a. Invert plate over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. Add 60 µL/well Working PreAmp Mixture. b. Cover plate with plate lid and incubate at 40 °C +/- 1 °C for 60 minutes.
5	Invert plate over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. Wash 3 times with 150 µL/well Wash Buffer.
6	Hybridize Amplifiers (Amps): a. Invert plate over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. Add 60 µL/well Working Amp Mixture. b. Cover plate with plate lid and incubate at 40 ± 1 °C for 60 minutes.
7	Invert plate over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. Wash 3 times with 150 µL/well Wash Buffer.
8	**IMPORTANT** Protect samples from light during this and all subsequent steps. Hybridize Label Probes (LPs): a. Invert plate over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. Add 60 µL/well Working Label Probe Mixture. b. Cover plate with plate lid and incubate at 40 ± 1 °C for 60 minutes.
9	Wash three times with Wash Buffer: a. Invert plate over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. Wash 3 times with 150 µL/well Wash Buffer. b. Remove (as above) Wash Buffer and add 150 µL/well 1X PBS. At this point, the plate is stable at room temperature for several hours.

To perform hybridizations:

Step	Action
10	Counterstain nuclei with DAPI solution: **! WARNING ! DAPI is a possible mutagen. Avoid contact with skin and mucous membranes.** **IMPORTANT** Protect from light. a. Prepare DAPI Working Solution by diluting DAPI Stock Solution (10 mg/mL) 1:10,000 in 1X PBS and briefly vortexing to mix well. For example, add 1 µL of stock DAPI solution to 10 mL 1X PBS. 7.2 mL is sufficient for one 96-well plate. b. Invert plate over appropriate receptacle and gently expel contents, then invert plate on a clean, dry paper towel for 1-2 seconds. Add 60 µL/well DAPI Working Solution. c. Incubate at room temperature for 1 minute. d. Remove (as above) the DAPI Working Solution and wash once with 150 µL/well 1XPBS. e. Add 150 µL/well fresh 1X PBS. The plate is ready for imaging. Alternatively, the plate can be stored at 4 °C for several days. Seal the plate with an adhesive seal and protect from light when storing at 4 °C. Note that after 2 days, the signal will drop by 50%.

To perform hybridizations:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>11</td><td>
Scan the plate on an imaging system by:
♦ Using DAPI, fluorescein and rhodamine channels, or by
♦ Visualizing under a fluorescent microscope using appropriate filter sets
We recommend the following:
♦ Magnify the image by 200-400-fold through the combined use of a 10X eyepiece and a 20-40X fluorescence objective with numeric aperture equal to or greater than 0.75
♦ Set exposure time to obtain optimal signal-to-background ratio. Normally, DAPI stain requires 10-50X less exposure time than for target RNA 1 and 2.
♦ Use the following table to determine the multi-bandpass fluorescence microscope filter sets to visualize signals. For the best viewing signal, set the gray scale or intensity level as follows:
– min = 2X above background outside of cell
– max = 4X above background outside of cell
– Adjust as necessary
<table>
<tr><th>Nucleic Acid</th><th>Fluorophore</th><th>Peak Excitation (nm)</th><th>Peak Emission (nm)</th><th>Purpose</th></tr>
<tr><td>Target RNA 1</td><td>Fluorescein</td><td>495</td><td>519</td><td>For detection of 1 copy/cell</td></tr>
<tr><td>Target RNA 2</td><td>Rhodamine</td><td>546</td><td>573</td><td>For detection of greater than 1000 copies/cell</td></tr>
<tr><td>Nuclear DNA</td><td>DAPI</td><td>358</td><td>461</td><td>For focusing and cell identification</td></tr>
</table>
</td></tr>
</table>